

Synergistic anticancer effects of bufalin and sorafenib by regulating apoptosis associated proteins

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Abstract. As one of the most recognized and well-known drugs for hepatocellular carcinoma (HCC), the antitumor effect of sorafenib against HCC remains to be improved. Bufalin has displayed an antitumor effect in HCC; however, whether the enhanced antitumor effect may be generated with their combined treatment remains unclear. Therefore, in the present study, their combined effects on HCC proliferation and apoptosis were investigated. It was revealed that either bufalin or sorafenib suppressed PLC/PRF/5 and SMMC-7721 cell proliferation in a concentration-dependent manner following incubation for 24 h, and the inhibitory effect was augmented with their combined treatment. The synergistic effect peaked in HCC cells treated with 20 nM bufalin and 10 μ M sorafenib. In addition, cell cycle and terminal deoxynucleotidyl transferase dUTP nick-end labelling assays revealed that bufalin also enhanced sorafenib-induced apoptosis. Colony formation assay demonstrated that combined treatment significantly suppressed HCC proliferation compared with treatment with either of them alone. Furthermore, B-cell lymphoma 2-associated X protein, caspase 7 and poly-(adenosine diphosphate-ribose) polymerase were upregulated in HCC cells with combined treatment. Taken together, the results of the present study revealed that the treatment of sorafenib combined with bufalin synergistically suppressed HCC proliferation and induced apoptosis. Therefore, bufalin combined with sorafenib may be a favorable treatment strategy for patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of most frequent causes of cancer-associated mortality worldwide (1). Despite the development of specific treatment strategies, the prognosis of HCC remains poor. The outcomes of patients with HCC are far from satisfactory even in patients undergoing surgical resection at early stages (2). For advanced HCC patients, sorafenib, a multikinase inhibitor, has been demonstrated to be one of the few effective drugs (3). Experimental evidence has shown that it suppresses tumor growth by its capacity to inhibit vessel formation and induces apoptosis by targeting vascular endothelial growth factor receptor, platelet derived growth factor- β and c-kit in a number of different cancer types (4,5). However, it only prolonged the survival of HCC by a few months (6). Thus, identification of drugs which strengthen the antitumor effect of sorafenib is greatly warranted.

Bufalin has been demonstrated to exert potent antitumor activities in a number of human cancer types (7). It suppresses tumor cell proliferation and angiogenesis, and induces apoptosis and differentiation in cancer cells (8-10). In addition, it has been reported to reverse multi-drug resistance in various types of cancers (11). Given that sorafenib and bufalin are potent antitumor drugs, the present study speculated that greater inhibitory effects may be generated in HCC with their combined treatment. In a previous study, their synergistic effect has already been confirmed by their inhibition of tumor cell proliferation and vessel formation (12). However, whether enhanced apoptosis would also be induced by their combined treatment requires further exploration.

In the present study, it was shown that bufalin promoted the inhibitory effect of sorafenib in tumor cell proliferation. Apoptosis was also increased greatly by bufalin in sorafenib-treated HCC cells. Furthermore, an *in vivo* study was conducted using the HCC cell line SMMC-7721 as it has been adopted to establish subcutaneous HCC tumors previously (13). It was demonstrated that the apoptosis rate was significantly increased in mice injected with bufalin. Ultimately, western blot analysis identified that B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), caspase 7 and poly-(adenosine diphosphate-ribose) polymerase (PARP) are

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important molecules responsible for enhanced apoptosis. In conclusion, the findings suggested that bufalin may promote sorafenib-induced apoptosis in HCC. Therefore, the combination of these drugs may have clinical utility as a favorable therapy in the treatment of HCC.

Materials and methods

Reagents and antibodies. Sorafenib (Selleck Chemicals, Houston, TX, USA) and bufalin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) and diluted to their working concentrations (sorafenib at concentrations of 2.5, 5, 10 μ M and bufalin at concentrations of 5, 10 and 20 nM). Antibodies against Bcl-2 (Abcam, Cambridge, UK; cat. no. ab692), Bax (Abcam; cat. no. ab32503), caspase 7 (Bioworld Technology, Inc., St Louis Park, MN, USA; cat. no. BS6544), caspase 8 (Bioworld Technology, Inc.; cat. no. AP0237), PARP (Bioworld Technology, Inc.; cat. no. BS70001) and GAPDH (Bioworld Technology, Inc.; cat. no. MB001) were also used.

Cell culture. PLC/PRF/5 and SMMC-7721 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare, Chicago, IL, USA) added with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare) and 1% penicillin/streptomycin at 37°C containing 5% CO₂. Cells were passaged when they reached 80% confluency and used after the third passage.

Determination of concentrations of sorafenib and bufalin that may achieve optimal synergistic effect. The combined index (CI) was calculated by the CalcuSyn software. CI>1 indicated an antagonistic effect, CI<1, indicated a synergistic effect and CI=1, indicated an additive effect (14).

Animals. The present study was approved by Fudan University Shanghai Cancer Center (Shanghai, China). A total of 24, 6-week old male Balb/c nude mice weighing 20 g were used in the present study, and were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were raised under the following pathogen-free conditions: Room temperature, 20°C; relative humidity, ~50%. The mice were given *ad libitum* access to food and water and maintained under a 12-h light/dark cycle. The mice were randomly divided into four groups: Control, sorafenib, bufalin and the combination, with six mice per group. Animals were raised in pathogen-free conditions and received humane care according to the principles of animal care issued by Fudan University (12). All experiments conformed to the stipulations of the Animal Experimentation of Fudan University. The mice were divided into 4 groups, those that were subjected to daily administration of either 10 mg/kg sorafenib (sorafenib group) via oral administration, 10 mg/kg bufalin (bufalin group) via intraperitoneal injection, a combination of both drugs (combination group) or saline via intragastric administration in the vehicle (control group). The treatment lasted for 16 days, following which the mice were sacrificed, and the tumors were obtained.

In vivo tumorigenicity assay. The mice were not fasted prior to the following treatments. A total of 1x10⁷ SMMC-7721 cells in a volume of 200 μ l PBS were injected into the right flank of each mouse to form subcutaneous tumors. When the volume of these subcutaneous tumors reached a size of 100-300 mm³, the mice were treated with intraperitoneal injections of 1 mg/kg bufalin (5 days/week), 30 mg/kg oral uptake of sorafenib (5 days/week), or a combination of the two drugs (intraperitoneal injections of 1 mg/kg bufalin combined with oral uptake of 30 mg/kg sorafenib). The control mice were injected with saline. The tumor-bearing mice were sacrificed following 16 days of treatments, and tumors were excised and subjected to apoptosis assays and hematoxylin-eosin (HE) staining. All procedures conformed to the ethical principles of animal experimentation as stipulated by Fudan University.

HE staining. Paraffin sections were baked at 70°C for 1 h, de-paraffinized in xylene, rehydrated in gradually varied alcohol, and the sections were treated with 3% H₂O₂ to neutralize endogenous peroxidase for 30 min. The antigen retrieval was also conducted. Following antigen retrieval, the sections were dipped into a Coplin jar containing Mayer's hematoxylin and agitated for 30 sec and 1% eosin Y solution for 20 sec with agitation at 25°C. The sections were dehydrated with two changes of 95% alcohol and two changes of 100% alcohol for 30 sec each.

Cell proliferation. Cell proliferation was determined using a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). HCC cells were plated in 96-well plates at a density of 5,000 cells per well. The cells were subject to treatment with 2.5, 5 and 10 μ M sorafenib and 5, 10 and 20 nM bufalin and the combination of both at these concentrations for 24 h at 37°C. Cell viability was measured following this incubation with drug for 24 h using the CCK-8 kit. The absorbance was measured at a wavelength of 450 nm with a microplate reader to determine the cell viability rate.

Different treatments of HCC cells. HCC cells were subject to 2.5, 5, 10 μ M sorafenib and 5, 10 and 20 nM bufalin and the combination of both at these concentrations for 24 h at 37°C.

Cell cycle assay. HCC cells were plated in 6-well plates at 2x10⁵ cells per well and were subjected to 10 μ M sorafenib, 20 nM bufalin and the combination of 10 μ M sorafenib and 20 nM bufalin for 24 h at 37°C. Then cells were trypsinized by 0.25% trypsin and then washed with PBS. A cell cycle assay was applied (Beyotime Institute of Biotechnology; C1052). Subsequently, cells were fixed in 70% methanol at 4°C for 2 h and stained with propidium iodide (PI) [which consisted of 0.5 ml staining buffer, 25 μ l PI staining reagent (20 X), 10 μ l RNase A (50 X)] for 30 min at 37°C. A flow cytometer was applied (FC500, Beckman Counter, Inc., Brea, CA, USA) to detect fluorescence at excitation wavelength of 350 nm. The multiCycle AV DNA Analysis software (version 306; Phoenix Flow Systems, San Diego, CA, USA) was adopted to perform analysis.

Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay. Tissue apoptosis was determined

with a TUNEL Detection kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in compliance with the manufacturer's protocol. Tumor samples from *in vivo* studies were rinsed in PBS and fixed in 10% paraformaldehyde/PBS for 20 min at 25°C. Samples were dehydrated in 70% ethanol, paraffin embedded, and sectioned (4- μ m). The slides were rinsed twice with PBS. A total of 50 μ l TUNEL reaction mixture was added on the sample and covered with parafilm during the incubation. The samples were incubated at 37°C for 1 h in a humidified chamber in the dark. Finally, the samples undergoing apoptosis were counted in three randomly chosen fields in a drop of PBS under a fluorescence microscope.

Evaluation of apoptosis via Hoechst 33258. Following treatment with sorafenib, bufalin or the combined treatment for 24 h, a total of 1×10^5 HCC cells were harvested and stained with Hoechst 33258 (Beyotime Institute of Biotechnology; C1011) for 5 min at 25°C. Apoptosis was detected using Olympus fluorescence microscope. The excitation wavelength was 350 nm, and the emission wavelength was 460 nm.

Evaluation of apoptosis via Annexin V and 7-ADD. Following treatment with sorafenib, bufalin or the combined treatment for 24 h, a total of 1×10^5 HCC cells were harvested and stained with the AnnexinV-PE/7-ADD or Annexin V-FITC/PI apoptosis detection kit (BD Biosciences). The fluorescence intensity was detected via flow cytometry (FC500, Beckman Counter, Inc.). Apoptosis rate was calculated by the proportion of apoptotic cells of the total cells, using 3 randomly chosen fields of view.

Colony formation assay. A total of 1.5×10^3 cells were seeded in each well of a 6-well plate and cultured in DMEM medium containing 10% FBS. Following 10 days from seeding, the cells were stained with crystal violet at 37°C for 20 min (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and counted under three fields of view. Gelcount 1.2 software (Oxford Optronix, Abingdon, UK) was applied to analyze the stained cells.

Western blotting. HCC cells were lysed in radioimmunoprecipitation assay buffer containing 1% proteinase inhibitor cocktail (Beijing Solarbio Science & Technology Co., Ltd.; R0020). The protein concentration was determined using a bicinchoninic acid assay. Proteins (20 μ g) were loaded were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes 5% skim milk was used as the blocking buffer to incubate the membrane for 1 h at 37°C. The corresponding proteins were detected with the primary antibodies against Bcl-2 (Abcam, Cambridge, UK; cat. no. ab692; dilution: 1:500), Bax (Abcam; cat. no. ab32503; dilution: 1:2,000), caspase-7 (Bioworld Technology, Inc., St Louis Park, MN, USA; cat. no. BS6544; dilution: 1:1,000), caspase-8 (Bioworld Technology, Inc.; cat. no. AP0237; dilution: 1:1,000), PARP (Bioworld Technology, Inc.; cat. no. BS70001; dilution: 1:1,000) and GAPDH (Bioworld Technology, Inc.; cat. no. MB001; dilution: 1:1,000) (as stated in *Reagents and antibodies*) then incubated with a horseradish peroxidase-conjugated secondary antibody (HAF008, dilution: 1:5,000; Novus Biologicals, LLC, Littleton, CO, USA), following which the proteins were visualized using an enhanced chemiluminescent

substrate (WBKLS0500; Merck KGaA). Semi-quantification of the blots was conducted using ImageJ software (version no. k 1.45; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed using SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA). Results were presented as the mean \pm standard deviation. The comparisons between two groups were made using Student's t-test. Multi-group comparisons of the means were made using one-way analysis of variance with the Student-Newman-Keuls used as a post hoc test. All experiments were repeated three times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Bufalin enhances the inhibitory effect of sorafenib on HCC cell proliferation. HCC cells were incubated with sorafenib, bufalin or sorafenib and bufalin in combination at different concentrations. Concentrations of 2.5, 5 and 10 μ M sorafenib and 5, 10 and 20 nM bufalin were used. The concentrations were chosen based around the IC₅₀ value of sorafenib and bufalin in both PLC/PRF/5 and SMMC-7721 cells. A CCK-8 assay was conducted to determine whether a synergistic effect existed between sorafenib and bufalin. As demonstrated in the results, the survival of HCC cells was reduced when treated with sorafenib at concentrations ranging from 2.5 to 10 μ M (Fig. 1A). Decreased proliferation was also observed in HCC cells treated with 20 nM or less bufalin (Fig. 1B). In addition, combination treatment led to decreased cell proliferation compared with either sorafenib or bufalin alone (Fig. 1C). As demonstrated in Fig. 1A and B, we have determined the optimal concentration of sorafenib (10 μ M sorafenib) and bufalin (20 nM bufalin) that would exert the most significant synergistic effect. Therefore, we have measured their combined effect according to these concentrations. The inhibitory effect of the combined drugs was more apparent as the concentration of sorafenib and bufalin increased, as shown from the CI/fractional effect curve (Fig. 1D and E). The combined treatment reduced cell proliferation significantly than either of them alone ($P < 0.05$). For PLC/PRF/5 and SMMC-7721 cells, some concentrations (CI < 1 when combined) were demonstrated to be synergistic whereas certain concentrations (CI > 1 when combined) were demonstrated to be antagonistic, as illustrated in Fig. 1D and E. As calculated by CI, the combination of sorafenib (10 μ M sorafenib) and bufalin (20 nM bufalin) was demonstrated to exert the most significant synergistic effect ($P < 0.05$).

Effect of the combination treatment on HCC cells. Cell cycle analysis of cells with different treatments was determined by examining the cells' DNA profiles following staining with PI. As shown from the cell cycle assay, SMMC-7721 cells treated with sorafenib and the combination of sorafenib+bufalin demonstrated similar proportions of cells in the different phases of the cell cycle compared with the control group, and the proportion of cells in the G1 phase stimulated with bufalin was smaller compared with the control group. In the G2 phase, SMMC-7721 cells treated bufalin made up the largest proportion whereas the proportion of SMMC-7721

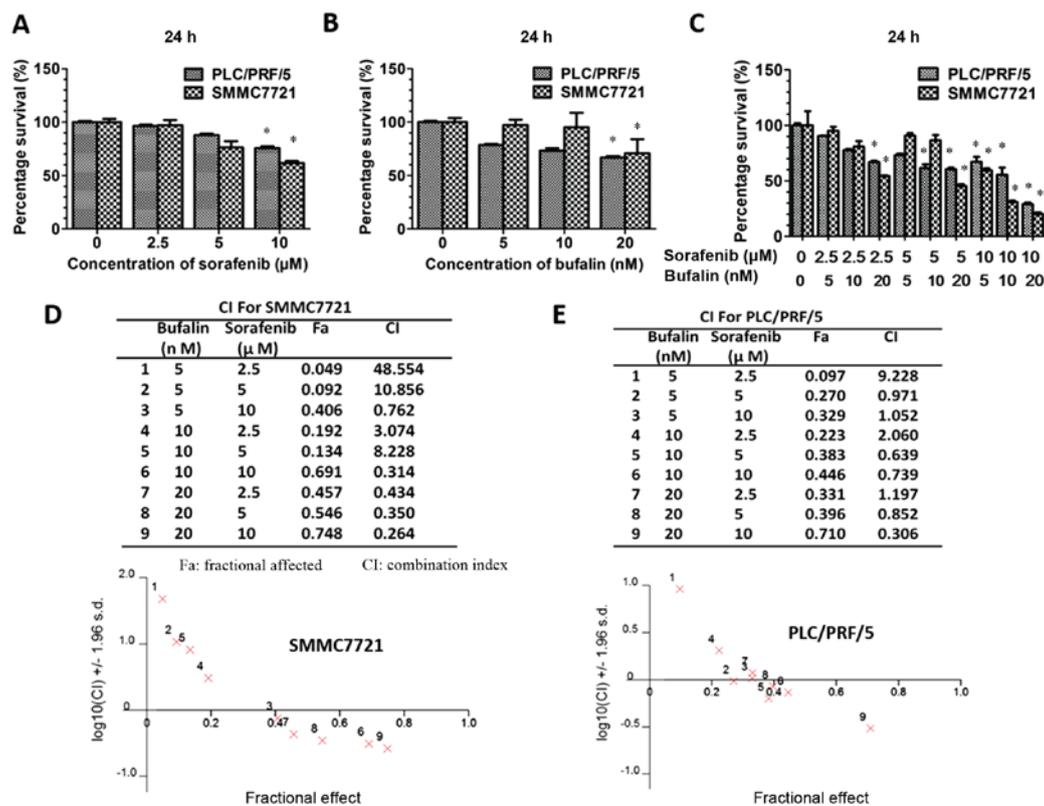


Figure 1. Bufalin enhances the inhibitory effect of sorafenib in HCC cell proliferation. (A) The survival rate of PLC/PRF/5 and SMMC-7721 cells treated with sorafenib at 0, 2.5, 5 and 10 μM for 24 h. (B) The survival rate of PLC/PRF/5 and SMMC-7721 cells treated with bufalin at 0, 5, 10 and 20 nM for 24 h. (C) The survival rate of PLC/PRF/5 and SMMC-7721 cells treated with the combined treatment of sorafenib and bufalin for 24 h at various concentrations. (D) The CI for SMMC-7721 cells treated with the combined treatment of sorafenib and bufalin for 24 h at different concentrations. (E) The CI for PLC/PRF/5 cells treated with the combined treatment of sorafenib and bufalin for 24 h at different concentrations. CI, combination index; HCC, hepatocellular carcinoma; Fa, fraction affected.

cells with the combined treatment was the smallest among all the groups. SMMC-7721 cells treated with bufalin made up the largest proportion among the four groups in S phase (Fig. 2A). With regard to PLC/PRF/5 cells, there was no significant difference in the proportion of cells treated with sorafenib, bufalin and the combined drug in all phases (Fig. 2B).

Apoptosis increases in HCC cells following a combination treatment with bufalin and sorafenib. As the present study demonstrated that the effect of the combined treatment was more evident in inhibiting HCC proliferation, the effect of combined treatment on apoptosis was investigated. Concentrations of 20 nM bufalin and 10 μM sorafenib were used to incubate HCC cells for 24 h. As shown in Fig. 3A, apoptosis was significantly enhanced in PLC/PRF/5 cells and SMMC-7721 cells with the combination treatment as compared with cells treated with the control, sorafenib and bufalin alone (Fig. 3A). The Hoechst 33258 staining was used to detect apoptosis in cells with different treatments. All the cells were stained with blue, but only the apoptotic cells were brightly illuminated as illustrated in Fig. 3A. For SMMC-7721 cells, the controls have the fewest cells undergoing apoptosis and the cells treated with the combined drugs underwent the most marked level of apoptosis. Sorafenib (10 μM) or bufalin (20 nM) promoted the apoptosis of HCC cells, as assessed by Annexin V/PI staining, while the combined treatment promoted

HCC cell apoptosis to a greater degree than single-agent treatment (Fig. 3B and C). These results suggested that bufalin may promote sorafenib-induced apoptosis in HCC cells.

Bufalin enhances the inhibitory effect of sorafenib in tumor clone formation. The effects of sorafenib and bufalin on HCC cell proliferation was further validated *in vitro*. Clone formation analysis was conducted. The number of viable cells was significantly fewer in the combined treatment group when compared with the mono-drug groups, indicating the enhanced suppressive tumor properties of the combined treatment in HCC ($P < 0.05$; Fig. 4A and B).

Bufalin enhances sorafenib-induced apoptosis and necrosis in mouse HCC tissues. The nuclei of all cells were stained blue and apoptotic nuclei were stained green. As shown in Fig. 5A, the level of apoptosis in the tissues from mice injected with either sorafenib or bufalin was higher than that of the control. The number of cells with fluorescent green was the highest in mice injected with the combined drug treatment. The apoptotic rate of each treatment group (control, sorafenib, bufalin and the combination) was then measured. The apoptotic rate of the sorafenib or bufalin only treatments was significantly higher than that of the control group; however, the highest rate was observed in mice injected with the combination treatment (Fig. 5B). In addition, hematoxylin and eosin (H&E) staining of tumors from mice with different treatments was

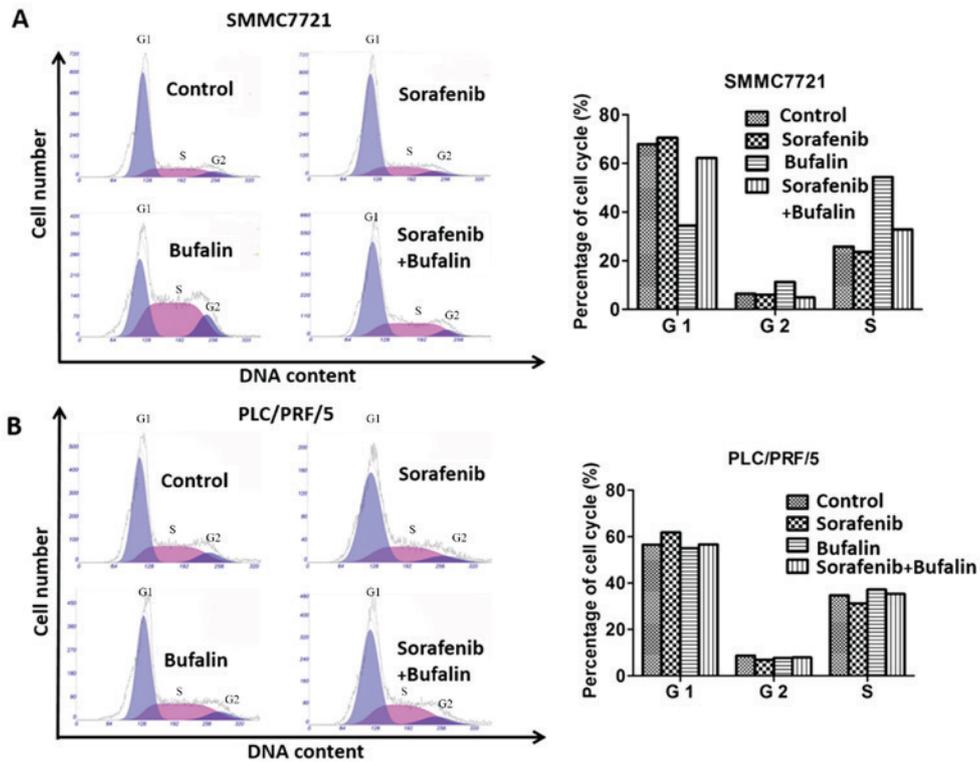


Figure 2. Effect of the combination treatment on HCC cells. (A) The percentage rate of SMMC-7721 cells at G1, G2 and S phase with the different drug treatments. (B) The percentage rate of PLC/PRF/5 cells at G1, G2 and S phase treated with the different drug treatments. All experiments were performed three times and the representative data are shown. HCC, hepatocellular carcinoma.

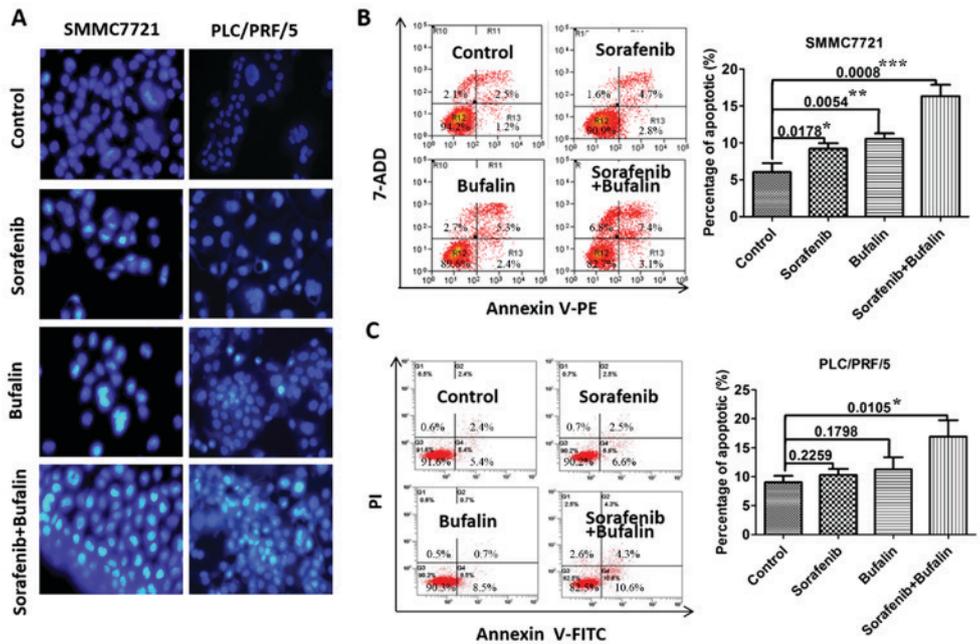


Figure 3. Bufalin synergizes with sorafenib to induce apoptosis in HCC cells. (A) The effect of sorafenib, bufalin and the combined treatment on SMMC-7721 and PLC/PRF/5 proliferation was measured using PI staining, magnification, x10. (B) SMMC-7721 cells were stained with an Annexin-V-PE/7-ADD apoptosis detection kit. The percentage of apoptosis was then measured. (C) PLC/PRF/5 cells were stained with a Annexin-V-FITC/PI apoptosis detection kit. The percentage of apoptosis was then measured. All experiments were performed three times and the representative data are shown with the corresponding P-values observed. All quadrants except for the lower-left represent apoptosis. *P<0.05, **P<0.01 and ***P<0.001, as indicated. HCC, hepatocellular carcinoma; PI, propidium iodide; FITC, fluorescein isothiocyanate.

observed. The results demonstrated that there were more areas of necrosis in tumors from mice treated with the combined agents as compared with the other groups (Fig. 5C).

Effects of the combination treatment on proteins associated with apoptosis. The expression levels of Bcl-2, Bax, caspase-7, caspase-8 and PARP proteins were measured

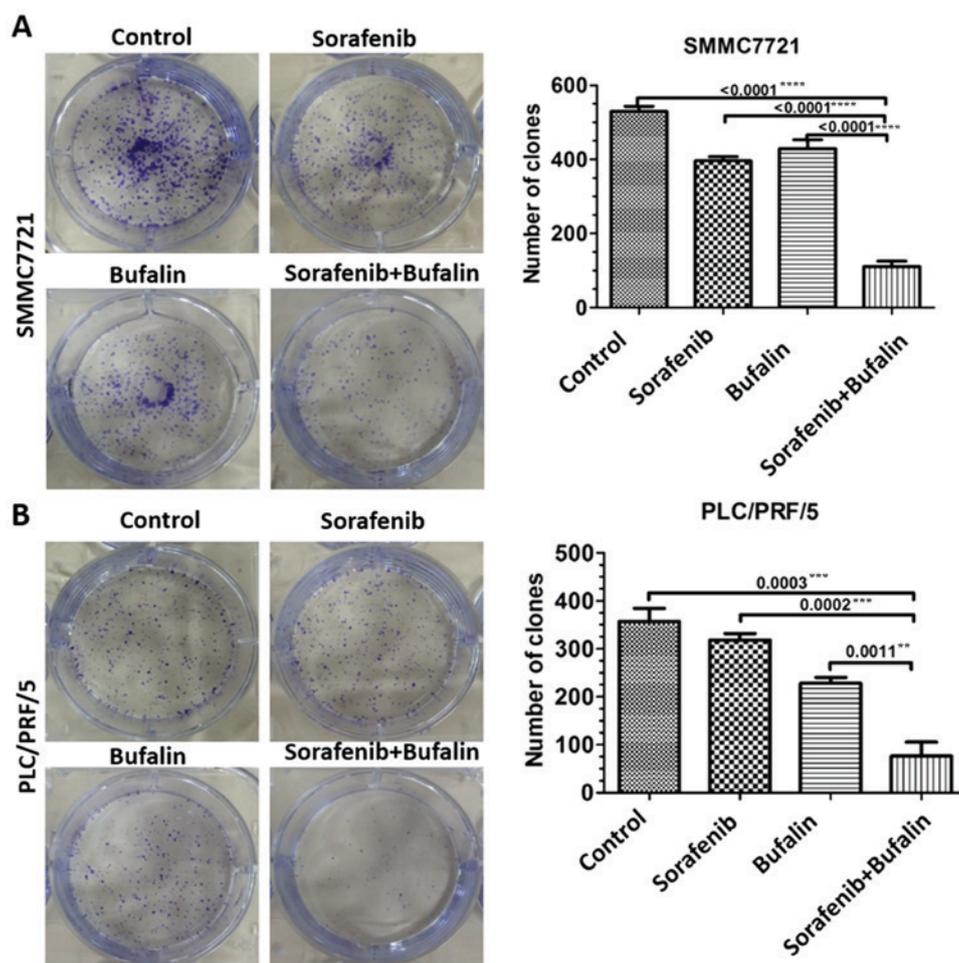


Figure 4. Bufalin enhances sorafenib's inhibitory effect in tumor colony formation. (A) Colony formation was evaluated in SMMC-7721 cells administered the different treatments. (B) Clone formation was also determined in PLC/PRF/5 cells following the different treatments. The numbers of clones in the different groups were then calculated. All experiments were performed three times and representative data are shown with the corresponding P-values observed. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$, as indicated.

in HCC cells following the different treatments. Sorafenib and bufalin treatment slightly decreased Bcl-2 levels when compared with the control; however, the combined treatment produced similar levels (Fig. 6). When compared with the control, Bax expression levels in HCC cells treated with sorafenib and bufalin did not show marked differences (Fig. 6). However, Bax expression was upregulated in cells treated with the combination of the 2 drugs, when compared with either drug alone (Fig. 6). Caspase-7 was upregulated in HCC cells treated with bufalin, as compared with the control cells; however, the difference was not statistically significant (Fig. 6). In HCC cells with the combined treatment, the expression of caspase 7 was upregulated as compared with the other groups (Fig. 6). In addition, caspase-8 was slightly increased in HCC cells treated with either bufalin or sorafenib alone, or with the combined-drug treatment, though not significantly so (Fig. 6). Both of the two bands (35 and 20 kDa) represent caspase 7, and both of the two bands (55 and 38 kDa) represent caspase 8. The doublet bands indicated different sized isoforms of caspase 7 and caspase 8. Furthermore, it should be noted that no significant difference in PARP levels were observed between the control and sorafenib and bufalin-treated cells. However,

PARP was significantly increased in cells with the combined drugs when compared with those treated with mono-drug, as demonstrated in Fig. 6. The 116 kDa band is the full length PARP and the 89 kDa is the cleaved PARP. In conclusion, the expression levels of Bax, caspase 7 and PARP were upregulated in HCC cells with the combined treatment of bufalin and sorafenib.

Discussion

The present study demonstrated that the combination treatment of sorafenib and bufalin was more potent in the inhibition of HCC cell proliferation when compared with either treatment alone. The combined drug also elicited increased cell apoptosis. Furthermore, apoptosis-associated proteins were altered in HCC cells with the combination treatment. Thus, the combination of sorafenib and bufalin may lead to enhanced HCC cell death.

Sorafenib, which has been proven to suppress tumor cell proliferation and angiogenesis, serves as the only recommended targeted therapy for advanced HCC (3-5). However, studies have revealed that the recurrence and progression is still high due to the development of drug resistance (15).

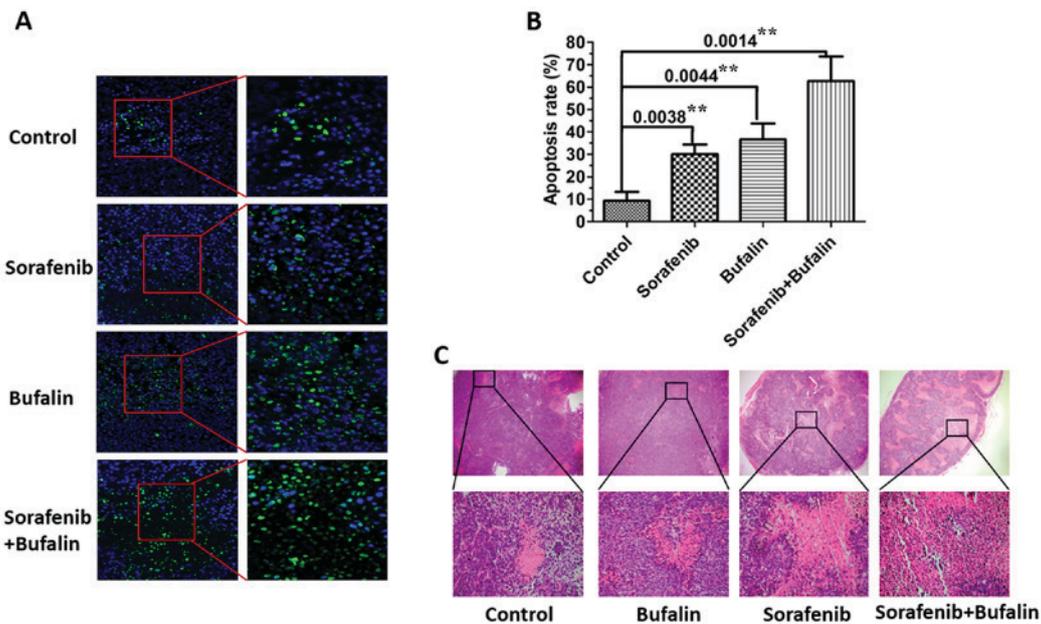


Figure 5. Bufalin enhances sorafenib-induced apoptosis in mouse HCC tissue. (A) The levels of apoptosis in the subcutaneous tumor tissues from mice treated with sorafenib, bufalin, the combined treatment and the control were detected. The number of apoptotic cells in HCC tissues from mice were analyzed via a TUNEL assay. TUNEL stained apoptotic cells were counted in three randomly selected microscopic fields, magnification, x10. (B) Apoptosis rates were calculated from apoptotic cells in HCC tissues. The corresponding P-values observed are also presented. ** $P < 0.01$, as indicated. (C) Representative hematoxylin and eosin images of subcutaneous tumors in mice injected with sorafenib, bufalin, the combined treatment and the control are shown, magnification, x4. Three independent experiments were performed and representative pictures are shown. HCC, hepatocellular carcinoma. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling.

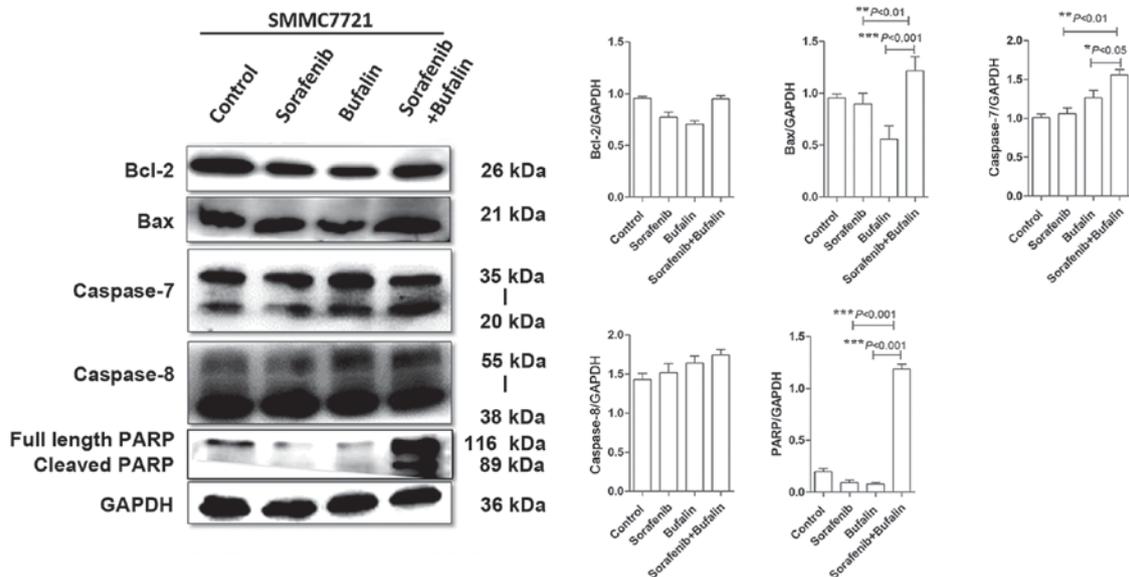


Figure 6. Effect of the combined treatment of sorafenib and bufalin on the expression of proteins associated with apoptosis. The protein expression levels of Bcl-2, Bax, caspase-7, caspase-8 and PARP were detected in SMMC-7721 cells treated with control, sorafenib, bufalin and combined treatment via western blotting and analyzed by ImageJ software. All experiments were performed three times. Representative blots and statistical analyses are shown with the corresponding P-values. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, as indicated. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; PARP, poly (adenosine diphosphate-ribose) polymerase.

Therefore, drugs that may strengthen the antitumor effect of sorafenib are warranted.

Bufalin has been widely investigated for its antitumor effects. Despite an increasing number of studies on bufalin, its antitumor mechanism is complex and remains to be further explored. It has been reported to suppress tumor cell proliferation and angiogenesis, induce apoptosis and cell

differentiation in many types of cancer (7-9). Indeed, the effect of bufalin relies largely on its concentration. Bufalin at low and high concentrations may exert different functions in promoting apoptosis or inhibiting metastasis. Studies have shown that bufalin may induce apoptosis via a number of different mechanisms. One previous study indicated that the intrinsic apoptotic pathway induced by bufalin is accountable

for reduced cell proliferation and tumor growth (16). In addition, the endoplasmic reticulum (ER) stress response regulated by the inositol-requiring enzyme signaling pathway may also contribute to bufalin-induced apoptosis (17).

As sorafenib and bufalin are potent drugs against HCC, the present study speculated that greater antitumor effects may be achieved by their combined treatment. Sorafenib and bufalin were tested to inhibit the proliferation of the PLC/PRF/5 and SMMC-7721 cell lines via a cell viability assay. As shown in the results, the suppressive ability of sorafenib increased when its concentration was $<10 \mu\text{M}$. Bufalin also demonstrated its inhibitive property against HCC cells in a dose-dependent manner. The present study determined the optimized concentration of sorafenib and bufalin in inhibiting HCC proliferation via CI indexes. The most significant proliferation rate was observed in HCC cells treated with $10 \mu\text{M}$ sorafenib and 20 nM bufalin.

Next, the present study determined the effect of the combination treatment on cell cycle arrest. The results demonstrated that the combination treatment (bufalin and sorafenib) did not have a synergistic effect on cell cycle arrest, as determined by a cell cycle assay.

Apoptosis, defined as programmed cell death, occurs in multicellular organisms under certain physiological and pathological circumstances, and is one of the approaches by which organisms maintain stability (18). A number of studies have shown that tumors have an infinite proliferative property and they also evade apoptosis (19-21). Dysfunction of apoptosis is one of the major mechanisms that leads to malignant tumors. Normal cells and tissues may undergo apoptosis once their microenvironment alters, ensuring the stability of physical activities (22,23). Accelerated proliferation of tumor cells may be attributed to escape from apoptosis (24). Therefore, identification of drugs that induce apoptosis is vital in tumor treatment.

The results presented in the present study revealed that sorafenib combined with bufalin may lead to significantly decreased survival in HCC cells. In addition, apoptotic properties were observed in sorafenib and bufalin (3,4). Therefore, it was predicted that bufalin and sorafenib may have enhanced apoptosis in HCC cells. Next, 20 nM bufalin and $10 \mu\text{M}$ sorafenib were adopted in the following experiments using CI index. It was demonstrated that sorafenib combines favorably with bufalin to induce increased apoptosis in HCC when compared with the untreated control, and only sorafenib or bufalin-treated cancer cells. The results showed that bufalin induced apoptosis in HCC and also accelerated sorafenib-induced apoptosis. The levels of apoptosis were the greatest in HCC cells that were administered the combination treatment.

Flow cytometry analysis revealed that the combination treatment of sorafenib and bufalin significantly promoted apoptosis in PLC/PRF/5 and SMMC-7721 cells. Sorafenib or bufalin induced marginal apoptosis in HCC cells, while the combined treatment induced significant apoptosis in HCC cells when compared with single-agent treatment. H&E staining of mouse tumors with different treatments also showed that necrosis was the most evident in the combined treatment group.

Apoptosis is associated with a series of signaling pathways. Multiple mechanisms on sorafenib-induced apoptosis in HCC cells have been reported. One previous study reported that tumors subjected to sorafenib incubation demonstrated

increased caspase-4 activation and CCAAT-enhancer-binding protein homologous protein upregulation (25). Another study demonstrated that sorafenib promoted apoptosis in PLC/PRF/5 and HepG2 cells (26).

To determine the underlying mechanism by which bufalin regulates the susceptibility of HCC cells to sorafenib, the expression levels of anti-apoptotic and pro-apoptotic proteins were measured in HCC cells with different treatments. The widely accepted mechanism of apoptosis includes the mitochondrial pathway, the death receptor pathway and the ER pathway (27-29). All of these signaling pathways may interact with each other to regulate apoptosis. A series of caspases are actively involved in apoptosis. The release of caspases varies according to different cell types and stimuli (30). Caspase 7 and caspase 8 were measured in HCC cells administered with different treatments in the present study. Caspase-8 is the initiating molecule of the extrinsic apoptotic pathway; it activates the effector caspase, which leads to cell apoptosis (31,32). It was revealed that caspase 7 and caspase 8 were elevated in the combination-treated HCC cells when compared with HCC cells treated with single-agent treatments, indicating that the combined treatment may lead to enhanced apoptosis.

Bufalin was shown to potently promote PARP and caspase-7 activation, while sorafenib was observed to slightly suppress Bcl-2. Together, these results indicated that bufalin contributed to sorafenib-induced apoptosis via the regulation of these proteins. In the context of the present study, it is particularly noteworthy that Bax, caspase-7 and PARP were all upregulated in HCC cells with the combination treatment.

In the present study, sorafenib and bufalin have been demonstrated to suppress the proliferation and promote the apoptosis of HCC cells. Bufalin was demonstrated to synergize with sorafenib to suppress HCC cell proliferation. The enhanced effect of these two drugs may be attributed to bufalin's capacity to promote the sorafenib-induced activation of Bax, PARP and caspase-8, as shown from the western blotting results of the present study. There are few studies exploring the effect of the combination treatment of bufalin and sorafenib on HCC apoptosis. Among the most common molecules participating in apoptosis, PARP and caspase-7 were revealed to be the most significantly altered.

A previous study demonstrated that Bax and Bcl-2, the main Bcl-2 family members, are intimately associated with HCC progression (33). This study revealed that bufalin altered the levels of Bcl-2 and Bax. Bcl-2 and Bax are two of the major proteins that regulate cancer progression. High Bcl-2 levels usually prevent tumor cells from undergoing apoptosis, while Bax promotes apoptosis by caspase induction via the activation of caspase-9 (34,35). The combination of sorafenib and bufalin significantly upregulated sorafenib-induced Bax expression; however, it only had slight effects on Bcl-2 expression. The results of the study may provide more of an understanding of the underlying mechanism of combined treatment of bufalin and sorafenib against HCC.

In the present study, it was revealed that the combination of sorafenib and bufalin was more potent in inducing cell death, which was accompanied by the increased induction of apoptosis, as compared with either drug alone. Thus, potentiation of apoptosis due to the combination of the two drugs may contribute to enhanced HCC cell death. Therefore, bufalin

may also serve as an apoptosis accelerator for sorafenib in HCC treatment. The results suggested that the combination of sorafenib and bufalin may be a potential therapeutic strategy for patients with advanced HCC. However, the toxicity and clinical efficacy of this combination therapy remain to be evaluated and therefore, further investigation is required.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HW performed the *in vivo* studies and designed the study. CZ performed the *in vitro* studies and wrote the original manuscript. HC conducted the western blot experiments and analysed the data. ZM designed the study and reviewed the article.

Ethics approval and consent to participate

The present study was approved by Fudan University Shanghai Cancer Center (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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